

Circulating immune complexes in systemic lupus erythematosus

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SUMMARY

Sera from twenty-one patients with systemic lupus erythematosus (SLE) were analysed for the presence of circulating soluble immune complexes by a sensitive and quantitative radioimmunoassay employing radioiodinated human C1q (C1q-deviation test). In twenty-five normal individuals the percentage of C1q inhibition was $2.64 \pm 4.45\%$. Eleven of the SLE patients had significantly elevated values, and the mean value for the group was $20.38 \pm 20.64\%$. The seven patients with renal disease had somewhat higher levels ($24.14 \pm 18.70\%$) than those without kidney involvement ($19.00 \pm 21.84\%$), and elevated levels of antibodies to native DNA also were associated with high levels of percentage of C1q inhibition. Both intermediate (7S–19S) and large ($> 19S$) complexes were present in the sera, and digestions with DNase and RNase indicated that antibodies to DNA and RNA accounted for only some of them. Serial studies in individual patients demonstrated the association of circulating complexes with, and often preceding, falling complement levels during disease activation.

INTRODUCTION

In systemic lupus erythematosus (SLE) tissue damage often results from the formation and widespread deposition of immune complexes, especially in the kidneys, the vascular system, the brain and the skin. The pathogenicity of circulating immune complexes largely depends on their ability to activate the complement system, thereby releasing vasoactive substances and mediators of inflammation (Cochrane & Koffler, 1973). The presence of DNA–anti-DNA immune complexes has been found to correlate with disease activity, and deposits of these complexes have been demonstrated by immunofluorescence techniques in affected tissues (Tan *et al.*, 1971; Keefe *et al.*, 1974; Atkins *et al.*, 1972; Reichlin & Mattioli, 1973). However, the existence of clinical SLE without significant renal involvement in the face of strong titres of antinuclear antibodies, indicates a role for other immune-complex systems, and suggests that *in vivo* formation of some kinds of complexes may be attended by relatively mild organ damage. Lupus sera are known to have activity against a number of other nuclear and cytoplasmic antigens.

In this study a sensitive radioimmunoassay has been used to obtain quantitative measurements of circulating soluble immune complexes in the sera of patients with SLE. The detection of such complexes provides a sensitive index of disease activity in these patients.

MATERIALS AND METHODS

Patients. Sera from twenty-one patients with SLE and twenty-five normal subjects were studied. All patients satisfied the ARA criteria for the diagnosis of SLE.

Preparation of human C1q. Human C1q was prepared by the method of Agnello, Winchester & Kunkel (1970). The C1q was precipitated from fresh human serum with DNA. After removal of the DNA by digestion with DNase the C1q was further purified by gel filtration through Sephadex G-200. The C1q was radiolabelled with 125 Iodine by the chloramine-T method

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(McConahey & Dixon, 1966), and then deep frozen at -70°C in aliquots in 0.1 M phosphate buffer at pH 7.2 until used. After thawing the C1q was centrifuged at 13,000 rev/min for 5 min prior to use to remove aggregated material.

C1q-deviation test. Quantitative measurements of complement-binding soluble immune complexes in sera were made using the C1q-deviation test (Sobel, Bokisch & Müller-Eberhard, 1975). The sheep erythrocytes used in the test were sensitized with subagglutinating amounts of rabbit anti-sheep haemolysin (Difco) at titres of 1:32 or 1:64 to obtain a final ^{125}I -labelled C1q uptake of 50% with normal human serum used as a control. The results are expressed in per cent inhibition of uptake (i.e. per cent deviation of ^{125}I -labelled C1q away from the erythrocyte-antibody artificial immune complexes by the test serum). Patients' sera were tested fresh without storage or freezing and thawing, and in duplicate (50 μl samples) following decomplexation at 56°C for 30 min, and at a 1:5 dilution in buffer as prescribed by the test (Sobel *et al.* 1975). The test was capable of detecting 8 $\mu\text{g}/\text{ml}$ of IgG aggregates added to normal human serum. In a group of twenty-five normal individuals the mean inhibition was $2.64 \pm 4.45\%$ (s.d.) with a range of 0–15%. These were healthy laboratory and hospital personnel and included thirteen men (ages 24–45 years) and twelve women (ages 24–50 years).

Enzyme digestions. Pancreatic deoxyribonuclease (Worthington Chemicals, Freehold, New Jersey) was incubated at 0.2 mg/ml with test sera for 2 hr at 37°C prior to performing the C1q-deviation test as described by Nydegger *et al.* (1974). Pancreatic ribonuclease (Sigma Chemical Co., St Louis, Missouri) was used similarly in test sera at 0.2 mg/ml for 2 h at 37°C .

Density-gradient ultracentrifugation. 0.3 ml of sera containing C1q-reactive material were diluted 3:2 and fractionated on linear 4.5 ml 10–40% sucrose density gradients in pH 7.2 phosphate-buffered saline by ultracentrifugation (Jerry, 1971). A 'cushion' of 0.3 ml of 70% sucrose was used at the bottom of the tube. The gradients were spun at 45,000 rev/min in an SW65 rotor for 6 or 12 hr at 4°C , and collected from below in 4 drop (0.18 ml) fractions for analysis. Total protein in the fractions was measured by the Lowry technique (Lowry *et al.*, 1951), and immunoglobulins were detected by precipitin analysis in gels (Jerry, 1971). The C1q-deviation test was done on the fractions using the gradient buffer (sucrose in pH 7.2 phosphate-buffered saline) as control.

Antiglobulin activity in serum fractions. Immune-complex-rich serum fractions from density gradients were analysed for the presence of anti- γ -globulin activity by a modification of the double counter-immunoelectrophoresis method of Phillips & Draper (1975). The complexes were dissociated by pretreatment with pH 2.8 citrate buffer, and then their components were separated by electrophoresis into strongly buffered agarose. Human Cohn Fraction II in phosphate buffer was applied to adjacent wells. After incubation at room temperature for 1 hr and then at 4°C for 4 hr, the appearance of precipitin lines indicated the presence of anti- γ -globulin activity in the immune complexes.

Other immunological studies. Serum immunoglobulins and C3 were quantitated by radial immunodiffusion (Hyland Immunoplates). Rheumatoid factor was detected by latex agglutination. Antibodies to native DNA were determined semi-quantitatively by counter-immunoelectrophoresis. Antinuclear antibodies were assayed by indirect immunofluorescence using rat kidney slices as tissue substrate.

RESULTS

C1q-reactive material in lupus sera

Sera from twenty-one SLE patients and twenty-five normal individuals were assayed for the presence of circulating immune complexes by the C1q-deviation test. Pertinent clinical and laboratory data for the SLE patients are summarized in Table 1. Seven of the patients had renal involvement, documented by renal biopsy in five, and the presence of significant proteinuria and active renal sediment in two. Results of the C1q-deviation test are shown in Fig. 1 and reported as percentage C1q-binding inhibition. Using 12% as the upper limit of normal (mean \pm 2 s.d.), eleven of the twenty-one SLE patients had high levels of C1q-reactive material in their sera. The mean percentage C1q inhibition (\pm s.d.) for this group was $20.38 \pm 20.64\%$, which is significantly higher than the normal values of $2.64 \pm 4.45\%$ ($P < 0.0001$). SLE patients with renal disease tended to have somewhat higher levels of percentage C1q inhibition ($24.14 \pm 18.70\%$) compared to those without kidney involvement ($19.00 \pm 21.84\%$). However, the difference is not statistically significant because of the large spread in values, although renal patients (five/seven) were more likely to have elevated levels than non-renal patients (six/fourteen).

The percentage C1q-inhibition values were compared with several parameters of disease activity (Table 1). Significant correlations were not found with levels of total γ -globulin or specific immunoglobulins, with random $\beta_2\text{C}$ values, or with erythrocyte-sedimentation rate. Rheumatoid factors were present in twelve of eighteen patients tested, but usually in low titres, and showed no correlation with the percentage of C1q-inhibition values.

All patients with SLE had antinuclear antibodies, but the titres did not correlate with levels of C1q reactivity. However, antibodies to native DNA (nDNA) were determined by counter-immunoelectrophoresis,

TABLE 1. C1q reactivity in the sera of SLE patients compared with clinical and laboratory data

Patient number	Age (years)	Presentation	Nephritis	Treatment*		ESR	γ -Globulin (G%)	Immunoglobulins (mg/100 ml)			β_2 C	ANF†	RF†	DNA antibody	Percentage C1q inhibition
				High	Low			G	A	M					
1	21	Fever, polyarthralgia, hemiplegia	+	+			2.89	2320	620	340	125	250	40	3+	53
2	60	Polyarthralgia			+		1.89	1952	405	270	50	50	160	0	0
3	39	Pleural effusion		+	+	51	1.95	1450	107	720	208	250	10	2+	64
4	45	Rash, anemia		+		50	2.88	2800	1600	212	55	250	20	2+	51
5	37	Polyarthralgias				35	1.51	1480	152	150	35	50	0	0	43
6	21	Fever, polyarthralgia, haematuria	+	+	+			2380	900	140	26	1250	20	3+	41
7	54	Polyarthralgia, semicoma		+		25					130	10			0
8	40	Fever, polyarthralgia, arrhythmia		+			1.51				250	40	40	2+	0
9	50	Polyarthralgias		0	0	21	1.62				149	50	0	0	0
10	39	Pleural effusion		+			1.59				55	250	20	3+	30
11	30	Rash, polyarthralgia, seizures	+		+		1.75	1500	205	162	54	250	20	0	7
12	29	Seizures		+		10	0.75	585	30	145	135	50	0	0	27
13	31	Acral gangrene		+		0	1.19	1200	305	98	90	50	0	0	0
14	35	Polyarthrits				36	1.88	1600	650	600	250	80			6
15	51	Polyarthrits		+		27	1.92	1800	490	268	98	1250	0	0	12
16	72	Amnesia, hemianopsia, coagulopathy		+		51	1.52				103	1250	20	2+	9
17	30	Cystitis, proteinuria	+		+			3200	560	700	95	10		0	34
18	33	Proteinuria		+	+	20	3.98				110	1250	40	0	15
19	50	Rash, polyarthralgia		+		27	1.64				77	250	0	2+	29
20	60	Fever, hemiplegia	+	+			1.22	1025	335	60	93	50		0	0
21	18	Fever, polyneuritis		+	+		2.32				250	640		0	7

* High = > 30 mg prednisone daily with immunosuppressive agents. Low = < 30 mg prednisolone daily alone.

† Reciprocal titres of antinuclear and rheumatoid factors.

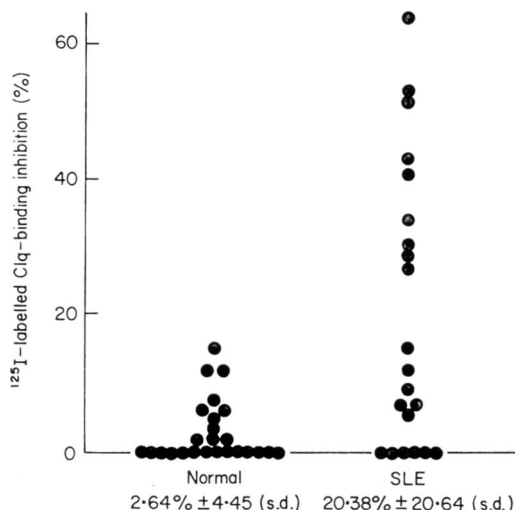


FIG. 1. Elevated C1q-inhibition values (percent) in patients with systemic lupus erythematosus (SLE).

and the intensity of the precipitin lines graded as strong (3+), moderate (2+), weak (1+) or negative (0) (Table 2). Strong precipitin lines to nDNA were associated with high levels of percentage C1q inhibition.

Enzyme digestion experiments

The association of high levels of antibodies to nDNA with elevated C1q values suggested that nDNA-antibody complexes contributed significantly to C1q reactivity. This contention was tested by determining the percentage C1q-inhibition values on sera before and after DNase digestion to remove such complexes. The effectiveness of digestion was followed in normal sera to which nDNA was added (40 µg/ml). Only three of the eleven patients tested showed a fall in C1q reactivity after DNase treatment of their sera. In two of these patients the fall was barely significant (7% and 5% in patients No. 1 and 4 respectively), although both had significant elevations of anti-nDNA antibodies. However, in patient No. 6 a striking fall of 40% (from 41% to 1%) in C1q reactivity occurred after DNase digestion. This individual had severe active renal disease with high titres of anti-DNA antibodies and very low levels of C3 (β1C, 26 mg/100 ml; normal, 125–225 mg/100 ml).

RNase digestion was performed on sera from nine patients, and depressed the C1q reactivity significantly in only one case (20% fall in patient No. 17). This 30-year-old woman had inactive renal lupus, and was suffering from acute lupus cystitis. There were no anti-nDNA antibodies in her serum, and her percentage C1q inhibition was 34%. DNase digestion of the same serum failed to alter the C1q reactivity.

TABLE 2. Relationship between C1q-reactive materials and antibodies to native DNA in lupus sera

Group	No. of patients	Antibodies to n-DNA*	Percentage C1q inhibition (mean ± s.d.)
(1)	4	Strong (3+)	46.00 ± 23.53
(2)	7	Moderate (2+)	28.71 ± 22.35
(3)	9	Negative	12.11 ± 12.49

Correlations: (1) vs (3), $P < 0.02$. (2) vs (3), P —not significant.

* Counter-immunoelectrophoresis.

Characterization of immune complexes by ultracentrifugation

Four lupus and two normal sera were fractionated by ultracentrifugation on linear sucrose-density gradients in neutral buffer. The position of C1q-reactive material was identified by performing the C1q-deviation assay on the gradient fractions. The typical profiles shown in Fig. 2 indicate that the C1q-reactive material in the lupus sera was present in the intermediate (7S–19S) and heavy (> 19S) regions of the gradient. This reactivity disappeared when the gradients were run at acid pH. None of the fractions of the normal sera showed significant C1q reactivity (> 5%).

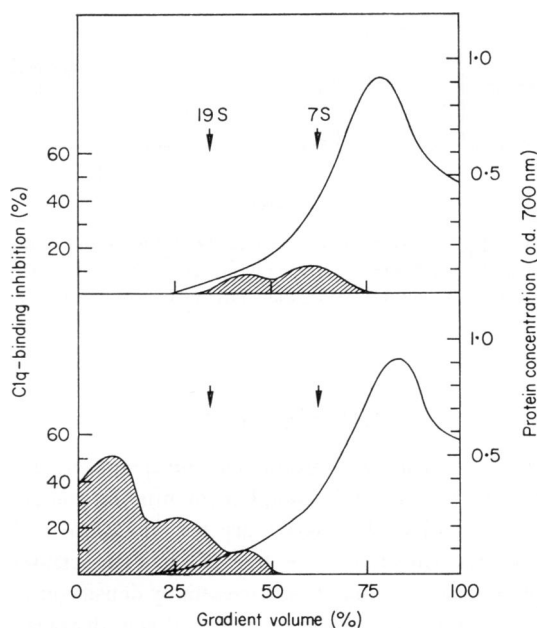


FIG. 2. Fractionation of two lupus sera by ultracentrifugation in linear 10–40% sucrose-density gradients at pH 7.2. C1q-reactive material (cross-hatched area) is seen in both intermediate (between 7S and 19S, patient 19, top) and heavy regions of the gradients (> 19S, patients 17, bottom). The solid line denotes total protein concentration.

Sequential studies of individual patients

Compared to random determinations, serial studies of circulating immune-complex levels in individual patients were much more informative. Two illustrative patients are shown in Fig. 3. The first patient (case 1) was a 21-year-old girl with previous severe renal lupus, who presented with high fever. In the absence of other clinical or laboratory evidence for reactivation of her lupus, the fever was ascribed to intercurrent infection, but it did not respond to antibiotics. At this time she had high levels of C1q-reactive material in her serum (53% C1q inhibition) despite normal levels of complement (C3). Shortly thereafter she developed sudden hemiplegia and coma due to lupus vasculitis. Only at this point was there a mild fall in serum complement (C3 to 91 mg/100 ml) and a rise in the ANF. The percentage of C1q inhibition had risen to 61%. She required massive doses of prednisone (300 mg daily) to control her neurological symptoms, and under the influence of this therapy there was a rapid fall in the percentage C1q-inhibition values to within the normal range (9%). Later, a further deterioration of her clinical status was accompanied by a severe rise in percentage C1q inhibition to 80 and a reciprocal fall in C3 levels to 80 mg/100 ml. She died a few days later with necrotizing pneumonitis.

The second patient (case 10) was a 39-year-old woman admitted with persistent pleural effusion. The ANF was elevated to 1/250, there were high levels of anti-n-DNA antibody in her serum (3+), and her C3 levels were low at 55 mg/100 ml. She had moderately elevated levels of C1q reactivity in her serum (30%). Following successful therapy with prednisone and azathioprine her percentage C1q-inhibition values fell to the normal range, and her C3 levels rose reciprocally to normal levels.

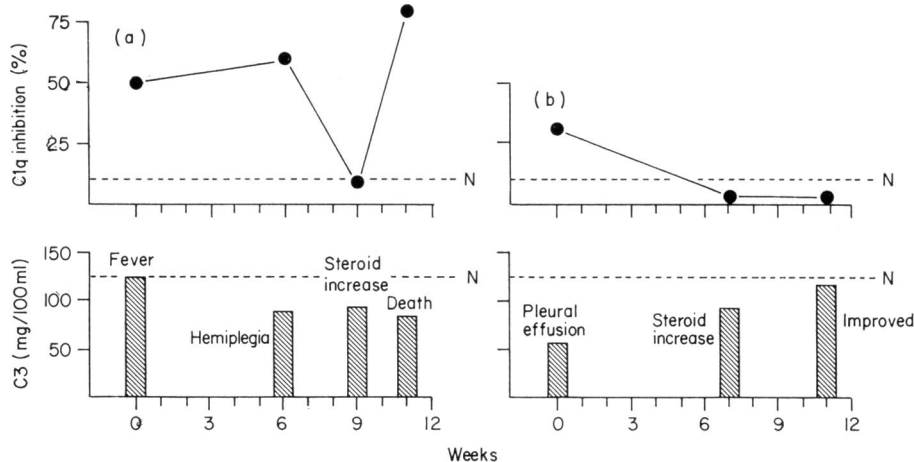


FIG. 3. Serial measurements of C1q-reactive material compared to C3 levels in two lupus patients. In patient 1 (a) elevated C1q-inhibition levels heralded reactivation of lupus activity before C3 levels fell. Both patients show a reciprocal relationship between C1q and C3 levels, and a fall in amounts of C1q-reactive material with corticosteroid therapy.

DISCUSSION

The C1q-deviation test provides a sensitive, reproducible and quantitative measurement of circulating immune complexes in patients with SLE. Only complement-binding complexes, however, are detected and some sources of error are possible. The assays are best done on fresh serum to avoid artifacts produced by γ -globulin aggregates. Interference by free DNA in the serum can be excluded by DNase treatment (Agnello *et al.*, 1969). Fractionation of positive sera by density-gradient ultracentrifugation, as illustrated here, reveals the size, immunoglobulin content and acid dissociability characteristic of C1q-reactive soluble immune complexes (Agnello *et al.*, 1970). Intercurrent infections may also produce soluble complexes or interference from endotoxin (Sobel *et al.*, 1975).

The presence of soluble immune complexes in SLE is well-documented (Cochrane & Koffler, 1973; Nydegger *et al.*, 1974; Agnello *et al.*, 1970, 1971; Theofilopoulos, Wilson & Dixon, 1976). Complexes are usually seen in sera from patients with renal involvement, particularly during active phases of the disease. Most of the patients with renal disease in this study were positive and as a group had the highest levels of C1q-reactive materials in their sera. However, a few SLE patients with only extra-renal manifestations and a mild form of disease showed high levels of C1q reactivity.

In view of the heterogeneity of the immune complexes detected and the potential multiplicity of antibody specificities that may be seen in SLE accompanying marked differences in disease severity, the C1q-reactive materials in some of these patients were characterized further. DNase-digestion experiments suggested that DNA-anti-DNA antibodies accounted for C1q reactivity in only three of eleven patients. Those that were positive had measurable free anti-DNA antibodies in their sera as well. The frequency of this type of immune complex may be higher since it is possible that some of the DNA complexes were protected from enzyme digestion by bound antibody (Parker & Osterland, unpublished observations). DNA containing complexes have been extensively studied (Tan *et al.*, 1971; Keeffe *et al.*, 1974; Atkins *et al.*, 1972; Agnello *et al.*, 1971), and their potential for severe damage is well-documented, particularly in renal lupus. Direct deposition of complexes in the kidneys with subsequent complement activation is the most likely mechanism.

The C1q-reactive fractions from patients 17 and 19 were found to possess anti-globulin activity as well. In patient 17 the anti-globulin was of the 19S type while in patient 19 it was 7S. Anti-globulin immune complexes are well known in SLE, and their cold insolubility may produce cryoglobulinaemia. Their association not only with SLE (Christian, Hattfield & Chase, 1963; Stastney & Ziff, 1969) but also with

the arthritis-purpura-nephritis syndrome (Meltzer *et al.*, 1966; Meltzer & Franklin, 1967), as well as their content of complement components and correlation with serum-complement diminution, have suggested a pathogenetic role in these diseases, especially the renal manifestations. However, the fact that rheumatoid patients have a minimal incidence of renal involvement despite the presence of rheumatoid factor-containing immune complexes (Luthra *et al.*, 1975; Zubler *et al.*, 1976), suggests that under certain but ill-understood conditions such complexes may have a low degree of pathogenicity for the kidney, while at the same time IgG antiglobulins may produce acute inflammation in joints (Winchester, Agnello & Kunkel, 1970; Winchester *et al.*, 1969a, b).

RNA and ribonucleoproteins constitute another important antigenic source in SLE (Reichlin & Mattioli, 1973; Parker, 1973; Schur & Monroe, 1969; Sharp, 1974). Little is known about their potential to form soluble immune complexes. It has been suggested that antibodies against non-DNA nuclear antigens may in fact protect the lupus patient against renal damage (Reichlin & Mattioli, 1972), since a patient with concomitant anti-DNA and anti-RNA antibodies had only minimal renal disease. Of nine patients studied here only one showed a decrease in C1q reactivity after RNase digestion. The girl had focal inactive glomerulonephritis and was quite similar to the two patients reported by Reichlin & Mattioli (1972). Fractionation of her serum on density gradients revealed the presence of C1q-reactive material in the > 19S region. It is likely that high-molecular-weight ribonucleoprotein-anti-ribonucleoprotein complexes existed in this patient.

In the patients reported here complexes containing DNA, RNA and rheumatoid factor were tentatively identified, but the nature of the C1q-reactive material in the remaining patients remains unknown. Other authors have been unable to identify many of these substances (Agnello *et al.*, 1971). Antibodies against other nuclear (SM) and cytoplasmic (RO) antigens could also be involved (Tan & Kunkel, 1966; Clark, Reichlin & Tomasi, 1969). Antibodies to both these antigens in lupus sera are capable of fixing complement (Clark *et al.*, 1969). Both are less than 150,000 in molecular weight and could account for some of the low- or intermediate-weight C1q-reactive material. Complexes involving C-type RNA viruses are also candidates (Mellors & Mellors, 1976).

The depletion of total haemolytic complement and of complement components in the serum (Schur & Sandson, 1968) and body fluids (Winchester *et al.*, 1970) is employed clinically as an indirect and early indicator of the presence of immune-complex-mediated tissue damage, as well as a useful guide for the suppression of disease activity by immunosuppressive agents. Serial studies in patients reported in this series confirmed a reciprocal relationship between serum C3 and C1q reactivity, and indicated that the C1q-deviation assay can give an earlier and more sensitive index of disease activity and response to immunosuppressive therapy by providing a direct measurement of complement-binding soluble immune complexes in the sera of SLE patients.

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